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Full Length Research Paper

Fine Structure and Energy Dispersive X-Ray Analysis (EDXA) of Tegumental Spines around the Acetabulum of Juvenile and Adult *Cyndiplostomum azimi* (Trematoda: Digenea)

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ABSTRACT

Energy dispersive X-ray analysis (EDXA) is a simple method based on X-ray emissions characteristic of chemical elements from the sample which is sorted by energy rather than wavelength using diffractive crystal. Recently, EDXA had been used to determine the chemical elements and their distribution in many helminthes to specify and correlated these variations with type species, type host and habitats; and to determine the effect of exponential growth of some helminthes on the concentration of some metabolically active elements inside the hosts. *Cyndiplostomum azimi*, as a model for digenea, was used to study the ultrastructural development of the tegumental spines around one of the attachment organs, the acetabulum, and correlate that with the concentration and distribution pattern of sulphur, calcium and phosphorous inside the spines, to define their possible function. Ultrastructure of the tegument and spines around the acetabulum of one, three and seven day old flukes revealed that the outer plasma membrane was corrugated during early development compared to that of seven day old (adult) flukes, where spines interrupted the syncytial distal cytoplasm that appeared as panels covered with less corrugated apical plasma membrane. Secretory bodies of different size, density and shapes were overcrowded during early development, but less dispersed in adults. The fibrous basal lamina that was very distinct in one and three day old flukes was not demonstrated in adults. The tegumental spines progressively developed and differentiated with the development of the flukes from young juvenile to the adult stage. Inclusion bodies of different size, density and shapes were numerous in the initial developmental stages and gradually became fewer in adults. The tegumental muscles which were poorly developed in juveniles were well differentiated in later stages of development. Energy dispersive X-ray microanalysis revealed that the concentration of the three elements under study; sulphur, calcium, and phosphorus decreased gradually with development to reach lower levels in seven day old flukes. Sulphur had the highest concentration in all stages of development followed by phosphorus then calcium.

Key words: *Cyndiplostomum azimi*, TEM, EDXA, Tegument, Spines.

INTRODUCTION

Energy Dispersive X-Ray analysis (EDXA) is a simple method based on X-ray emissions characteristic of a chemical element from the sample which issorted by energy rather than wavelength using a diffractive crystal. High energy electrons from the scanning electron microscope cause the excitation of X-rays whose energies and relative abundance depend upon the composition of the sample (Cazaux 1984; Johnson 1993; Vaughan 1989).

In conjunction with electron microscopy, this technique has been applied for many purposes like the quality control of products and fish diseases (Heckmann 1997) and examination of numerous species of animals to further understand the nature of protoplasm and animal organs (Smith and Richards 1991).Recently, EDXA has been usedto determine the chemical elements

and their distribution in many helminthes to specify and correlated these variations with type, type host and habitats (Heckmann et al. 2007, 2010, 2012 a,b; Radwan et al. 2012) and to determine the effects of exponential growth of some helminthes on the concentration of some metabolically active elements inside the hosts (Wraniczetal. 1996).

X-ray microanalysis of both the surface and internal parts of Acanthocephaluslucii (*Acanthocephala*) hooks demonstrated the presence of calcium, magnesium, phosphorus and sulphur (Brázová et al. 2014).

Similarly, EDXA of whole hooks of Echinococcus granulosus protoscoleces demonstrated the presence of sulphur and trace quantities of phosphorus. X-ray near-edge absorption spectra resembled those of cystine, feather and hair and showed the sulphur to be predominantly in the form of disulphide linkages (Smith and Richards 1991). X-ray elemental analysis was used to investigate the composition of the anchors, hooks and bars of the monogenoid Gyrodactylus *spp*. (Kayton 1983). *Cyndiplostomum azimi*, was used as an model for a digenean aiming to study the ultrastructural development of the tegumental spines on the ventral surface around the ventral sucker and correlate that with the concentration and distribution pattern of sulphur, calcium and phosphorous inside the spines, to define their possible function.

MATERIALS AND METHODS

Encysted metcercariae of *Cyndiplostomum azimi* were collected from the muscles of naturally infected *Clariasgareipinus*. Ten albino rats (*Rattus albus*) were experimentally infected orally with encysted metacercariae. Juvenile and adult *C. azimi* were collected from the fore intestine of rats; one, three, and seven day post infection. Worms were fixed in4% glutaraldehyde and processed for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) with X-Ray microanalysis unit attached.

For transmission electron microscopy, specimens were post fixed in 1% Osmium tetroxide (OsO4), dehydrated and embedded in Spur resin. Semi (1µm thick) and ultrathin (80nm) sections were stained with lead citrate and uranyl acetate and examined by a JEOL 1200 CX electron microscope at an accelerating voltage of 80KV. The ventral surface of the tegument surrounding the acetabulum of different stages of development were thoroughly examined and photographed by digital imaging camera attached to a computer.

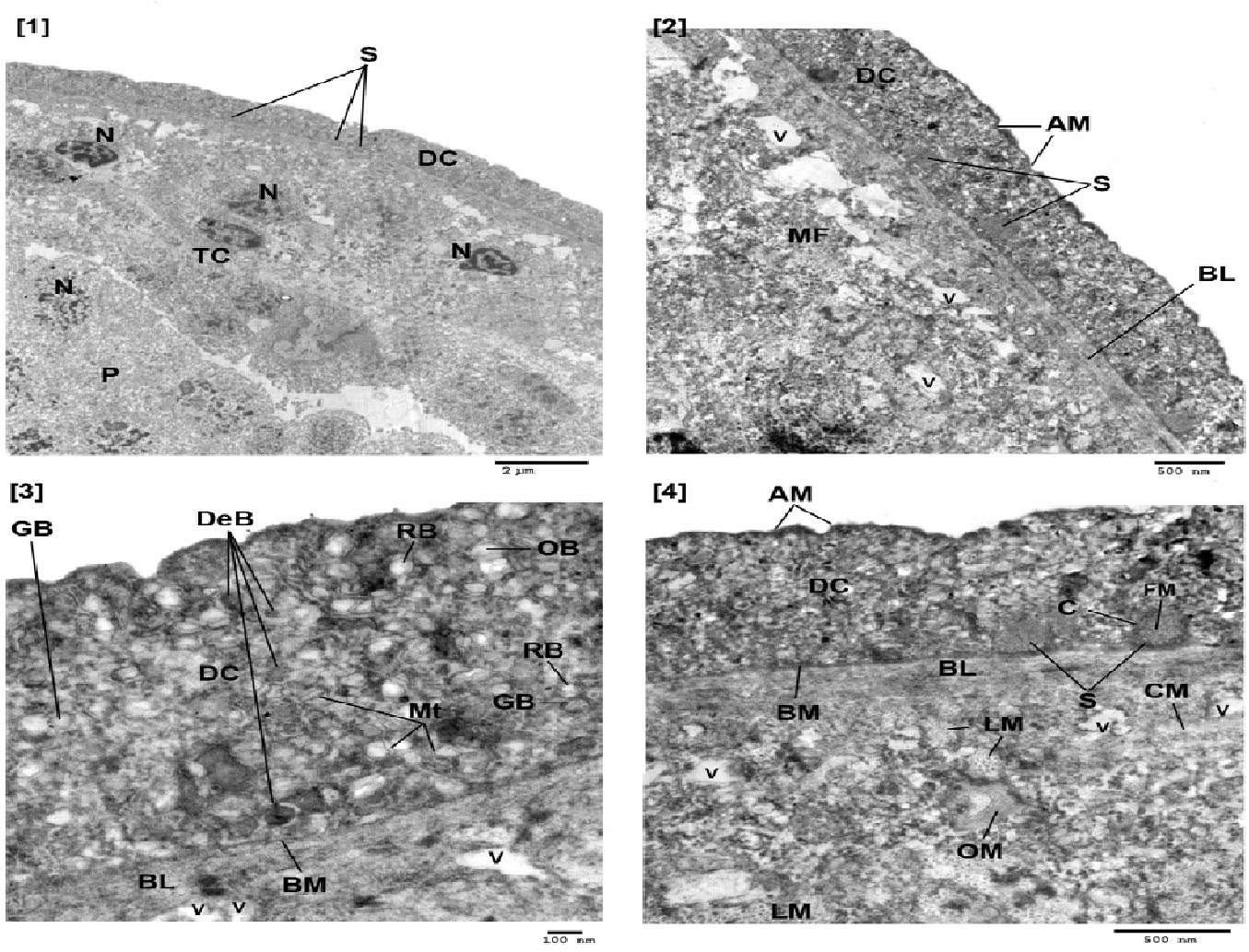
For X-ray microanalysis, standard methods for SEM preparation (Lee, 1992) were used. Mounted specimens were gold coated for 30seconds (approximate gold coating thickness of 20nm). Specimens were examined in a JOELJXA-840A electron microscope in combination with INCA X-sight X-ray analyzer. Samples were imaged at 30KV. EDXA scans were completed for triplicate specimens to examine spines around the ventral sucker of three developmental stages: one, three and seven day old. Weight percent (wt.%) of each chemical element sulphur, calcium, phosphorous of the spine was recorded and analyzed for probability using SPSS computer program.

RESULTS

Ultrastructure of the Tegument and Tegumental Spines around the Ventral Sucker

Ultrastructure of the tegument covering the ventral surface surrounding the acetabulum of one day old *C.azimi* reveals that the apical plasma membrane is corrugated (figures 2 and 4). The distal cytoplasm is overcrowded with ovoid and rounded shaped bodies of different size and electron density. Some of these bodies have dense margins, while others include dense or granulated material (figure 3). Microtubules are distributed in between these bodies (figure 3). The tegumental spines are represented by small ovoid opaque bodies in the basal part of the distal cytoplasm, resting on the basement membrane (figures 1, 2 and 4). Longitudinal sections of the spines revealed opaque fibrillar medulla surrounded by electron dense cortex that becomes thicker at the basal layer of the spine (figure 4). The trilaminated basal basement membrane underlines the distal cytoplasm (figures 3 and 4). A distinct fibrous basal lamina extends between the basement membrane and the poorly developed tegumental muscles (figures 2, 3 and 4). The tegumental muscles are represented by few longitudinal, circular and oblique muscle fibers (figure 4). Numerous small vacuoles are lined underneath the basement plasma membrane (figures 3 and 4). In addition, vacuoles of different size and shape are scattered in between the muscle fibers (figures 2 and 4). The tegumental cells are irregular in shape, numerous and enclose large ovoid nuclei and are embedded in the parenchyma (figure 1).

Figures 1-15 shows the transmission electron micrographs of the tegument around the ventral sucker of juvenile and adult *Cyndiplostomum azimi.*



Figures 1-4: Transmission electron micrographs of one day old flukes

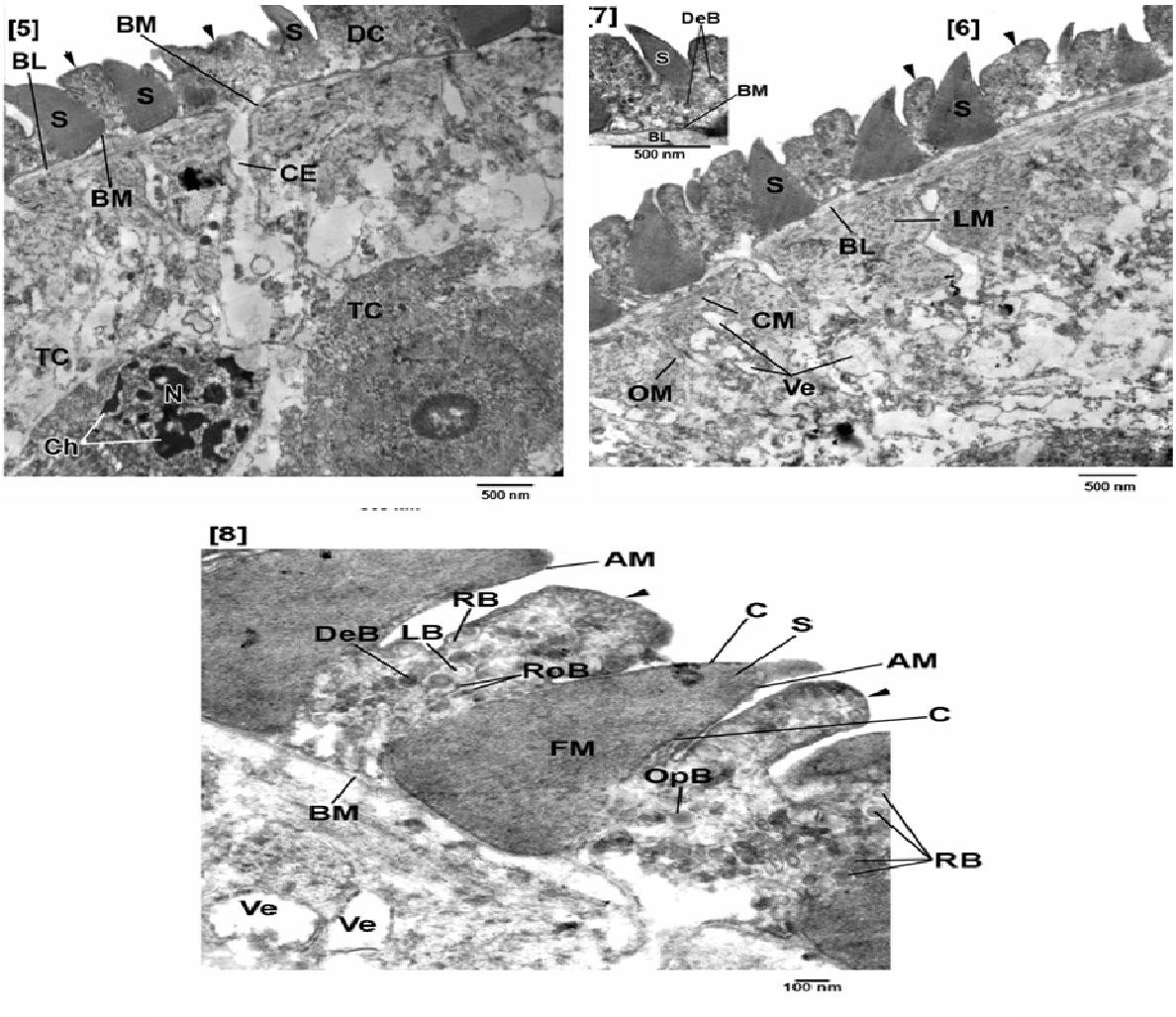
Figures legends

Figure1: showing distal cytoplasm, tegumental spines, tegumental cells, nuclei and parenchyma; **Figure 2:** showing apical plasma membrane, distal cytoplasm, small ovoid opaque tegumental spines, fibrous basal lamina, poorly developed muscle fibers and vacuoles; **Figure 3:** High magnification of distal cytoplasm shows ovoid bodies, round bodies, granulated bodies, dense bodies, microtubules, trilaminated basement membrane, fibrous basal lamina and vacuoles; **Figure 4:** showing apical plasma membrane, distal cytoplasm, longitudinal sections of tegumental spines, medulla, cortex, basal basement membrane, basallamina, vacuoles, circular muscle fibers, longitudinal muscle fibers and oblique muscle fibers.

Abbreviations in all figures

AM: apical plasma membrane; **B:** base; **BL:** basal lamina; **BM:** basement membrane; **C:** cortex; **Ch:** chromatin; **CM:** circular muscle fibers; **CT:** cytoplasmic extensions; **DC:** Distal cytoplasm; **DeB:** dense bodies; **DG:** dense granules; **ER:** endoplasmic reticulum; **FM:** fibrillar medulla; GB: granulated bodies; **LB:** lucent bodies; **LM:** longitudinal muscle fibers; **M:** mitochondria; **MF:** muscular fibbers; **Mt:** microtubules; **N:** nucleus; **OB:** ovoid bodies; **OM:** oblique muscle fibers; **OpB:** opaque bodies; **P:** parenchyma; **Pi**: pits; **RB:** round bodies; **RoB:** rod-shaped bodies; **S:** spines; **TC:** tegumental cell; **V:** vacuoles and **Ve:** vesicles.

Ultrastructural study of three day old *C.azimi* revealed that the apical plasma membrane although less corrugated, yet infolded deeply such that the distal cytoplasm appears as cytoplasmic panels separated by well-developed spines (figures 5, 6 and 8). The distal cytoplasm encloses bodies of different size, shape (rounded and rod-shaped) and density (lucent, dense and opaque) (figure 8). Some bodies possess dense margins and others had dense cores (fig. 8). Electron dense bodies are clearly seen near the base of the spines (figure 7). The tegumental spines are dense conical shaped bodies with broad base and posteriorly directed shafts that protrude above the tegumental surface in the form of pointed tips (figures 5- 7 and 8). The dense cortex and fibrillar medulla are well differentiated (figure 8). The basal part of the spine rests on the tegumental basement membrane, while the tip is covered with the apical plasma membrane (figure 8). The trilaminated basal plasma membrane is underlined by a fibrillar basal lamina (figures 5-7). The trilaminated basal basement membrane underlines the distal cytoplasm extending proximally to reach the perikarya (figure 5). The tegumental cells (perikarya) have ovoid nuclei with dense chromatin extend by cytoplasmic extensions of the distal cytoplasm (figure 5). Tegumental musculature is differentiated into bundles of longitudinal, circular and oblique muscles fibers that underline the fibrillar basal lamina (figure 6). Membrane bound vesicles of irregular shape are scattered in between the muscular layers (figures 6 and 8).



Figures 5-8: Transmission electron micrographs of three day old flukes

Figures legends

Figure 5: showing digitiform cytoplasmic panels (arrowheads), conical dense shape tegumental spines with pointed tips, trilaminated basal plasma membrane, fibrillar basal lamina, cytoplasmic extensions, tegumental cells, nuclei have dense chromatin; **Figure 6:** showing tegumental spines, digitiform cytoplasmic panels, fibrillar basallamina, circular muscle fibers, oblique muscle fibers, longitudinal muscle fibers and membrane bound vesicles; **Figure 7:** showing dense bodies near the base of the tegumental spine, basal plasma membrane and fibrous basal lamina**; Figure 8:** High magnification of distal cytoplasm shows apical plasma membrane, digitiform cytoplasmic panel (arrowheads), round bodies, rod-shaped bodies, dense bodies, lucent bodies, opaque bodies, tegumental spines with dense cortex and fibrous medulla, basement membrane and dense membrane bound vesicles.

Ultrastructural study of seven day old *C.azimi* reveals that the trilamenated apical plasma membrane is corrugated (figures 9a, 9b and 10) and coating the entire outer surface of the distal cytoplasm which appears as cytoplasmic panels, irregularly disrupted by spines(figures 13 and 14). Inclusion bodies of different sizes and shapes are seen scattered within this layer. Although dense bodies are less illustrated followed by opaque ones, yet lucent bodies are numerous sometimes overlapping, giving a reticular appearance, with tegumental spines entangled within (figures 10, 12-15) Some of the inclusion bodies have clear matrices while others enclose dense granular content (figure 12). Ovoid bodies with opaque core and dense periphery are occasionally encountered (figure12).Distal cytoplasm encloses some dense mitochondria (figures 12 and 14).

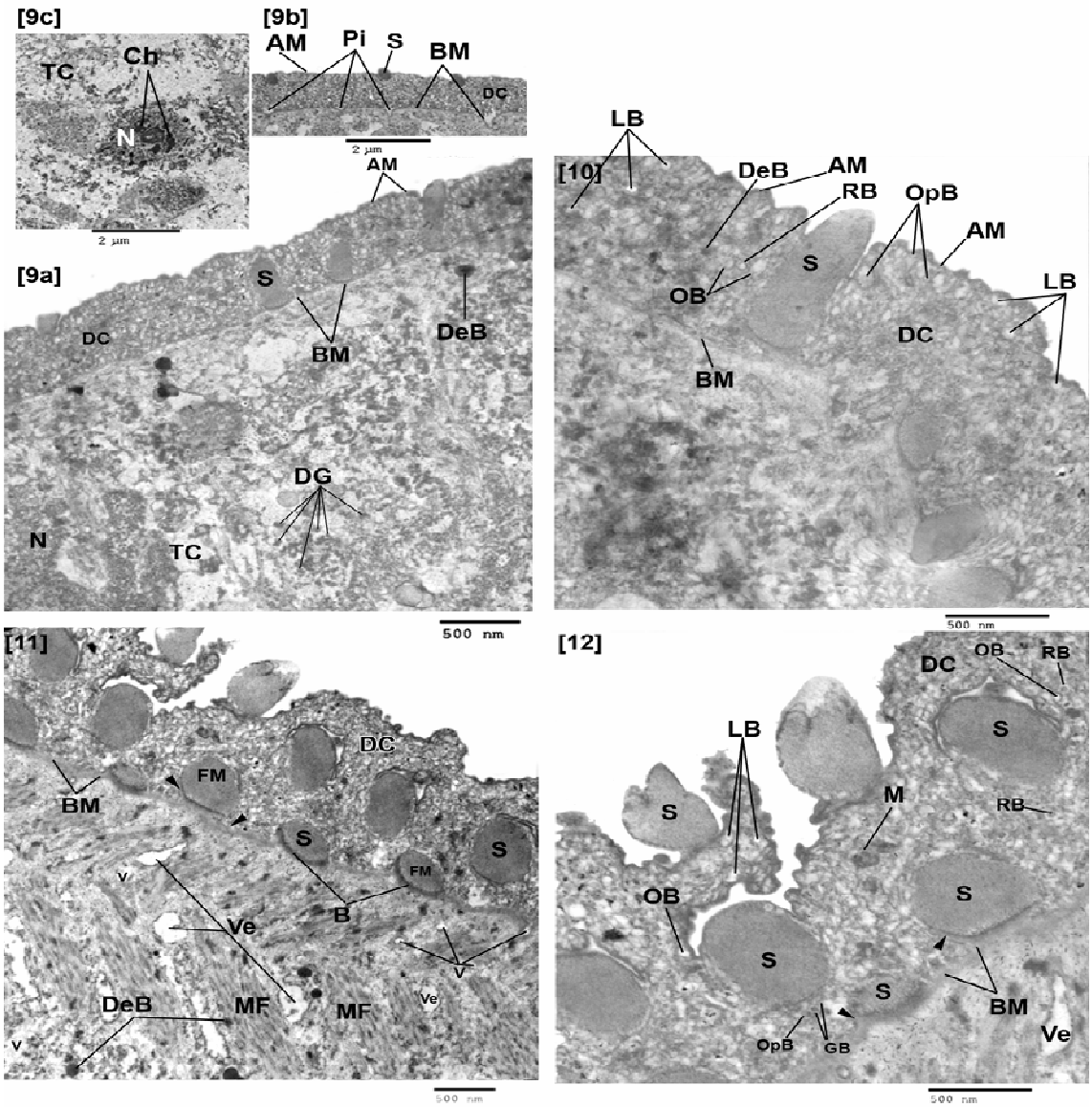
Tegumental spines are embedded within the syncytial cytoplasm and appear in different forms, mostly, with their rounded, broad or digitiform tips protruding above the apical plasma membrane (figures 10, 13 and 14). Sometimes, the tip of the spine appears covered by an extension of the distal cytoplasm (figures 11, 12). The base of the tegumental spines restsand is often embedded, in a dense granular infolding of the basement membrane (figures 10-12). In most spines the free distal edge of the spine appears covered by the apical plasma membrane (figures 13 and 14). The body of spines consists of a homogenous fibrillar matrix with electron-dense base (figures 11, 14 and 15).

In certain areas, spines appear crowding at different levels, some are hanging in the distal part of the syncytial cytoplasm and others are entangling in the middle or resting on the basal plasma membrane. These different positions may result from the crowding and position of the spines within the distal cytoplasm in relation to the orientation of cutting of the section (figures 11 and 12). In some areas, digitiform spines appear alternating with panels of distal cytoplasm. The base of these panels, often enclose

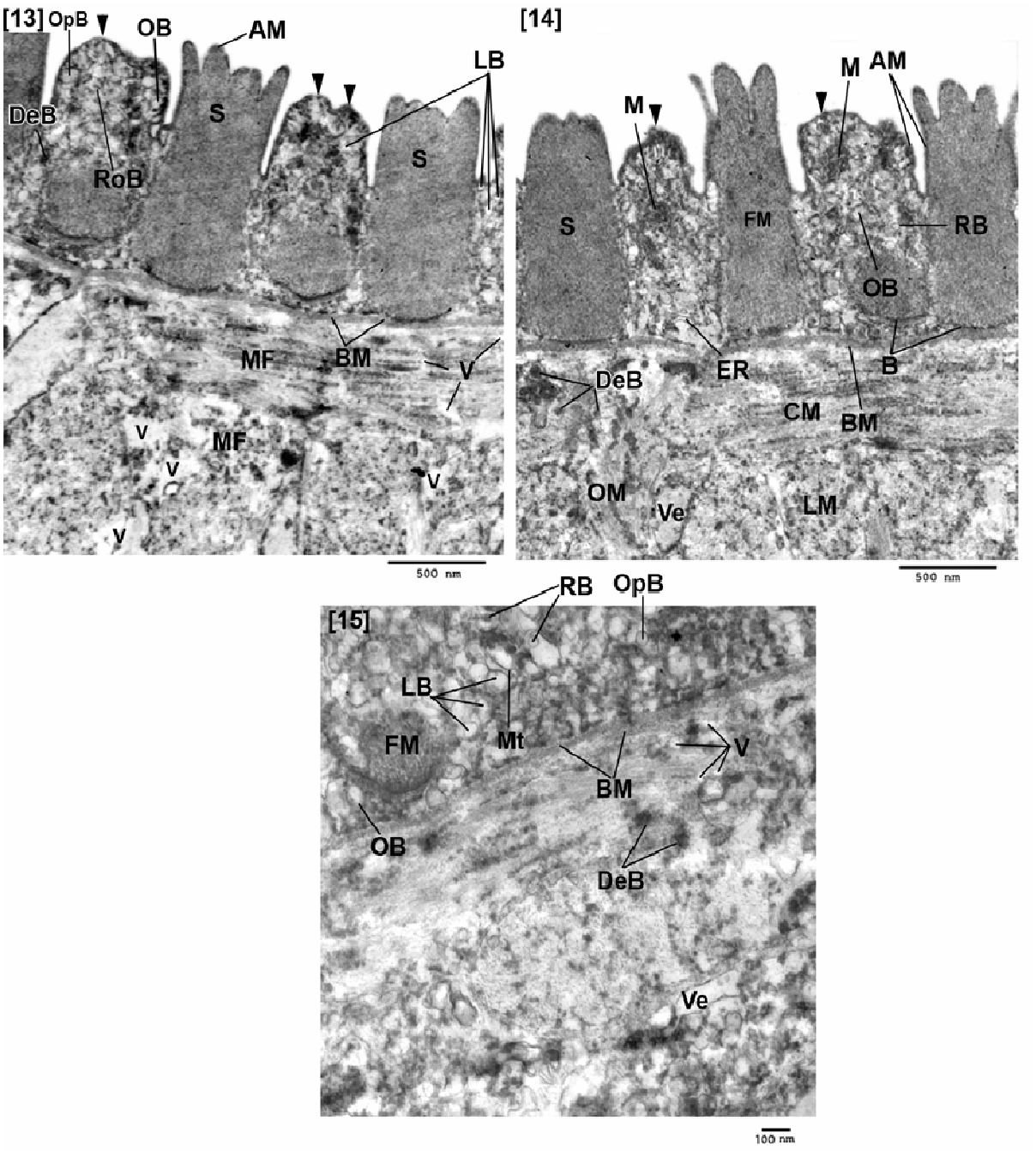
electron opaque material that resembles the base of the spine together with vesicular bodies and cisternae of endoplasmic reticula (figures 13 and 14).

The distal tegumental cytoplasm is underlined by a dense trilaminated basement membrane that is straight in some parts (figure 13) or extend deep in the distal cytoplasm to line the base of tegumental spines (figures9 a, 10, and 12); in other parts it is not straight in some parts forms pits and infoldings that extend at irregular intervals(figure 9b). Tegumental cells possess large, ovoid to round nuclei enclosing dense irregularly segmented chromatin(figures 9a and c).It is worth mentioning that the perikarya in this region appear filled with electron dense granules (figure 9a). The distal cytoplasm is underlined by two tegumental muscle layers; circular and longitudinal. The circularly oriented muscle fibers are grouped in bundles, whereas the longitudinally oriented fibers are densely distributed adjacent to the circular fibers. In addition, obliquely directed muscle fibers appear through the sections (figure 14). In some parts, muscles are set in a feather-like arrangement (figure 11).

Numerous membrane bound vesiclesenclosing dense material are located in between and underneath the muscle bundles. This region exhibits many dense bodies that are most probable secretory (figures 9a, 11, 14 and 15). The ultrastructure of the tegument of seven day old *C. azimi* exhibited variations in different areas. Some are richer in having spines and muscle bundles than others (figure 11). In some areas, small lucent vesicles are observed near the tegumental surface (figures 11 and 12); while in others, larger vesicles are detected in between the muscular layers (figures 11 and 15). Moreover many vacuoles with different size could be detected between the muscle bundles (figures 11, 13 and 15). In other areas small microtubules are detected in the distal cytoplasm (figure 15).



Figures 9-12: Transmission electron micrographs of seven day old flukes



Figures 13-15: Transmission electron micrographs of seven day old flukes

Figures legends

Figure 9a: showing trilamenated apical plasma membrane, corrugated distal cytoplasm, tegumental spines, dense trilaminated basal plasma membrane, dense bodies tegumental cell with large round nucleus and electron dense granules; **Figure 9b:** showing apical plasma membrane, distal cytoplasm, spines and trilaminated basement membrane with pits; **Figure 9c:** High magnification of a tegumental cell with large ovoid nucleus and dense irregularly segmented chromatin; **Figure 10:** showing highly corrugated trilamenated apical plasma membrane, distal cytoplasm, round bodies, oval bodies, dense bodies, opaque bodies, lucent bodies, tegumental spine with rounded tip and basal plasma membrane; **Figure 11:** showing longitudinal sections of tegumental spines at different levels, distal cytoplasm, infoldings of basal membrane (arrow head) homogenous fibillar matrix, electron dense base, feather-like arranged muscle fibers, membranous bounded vesicles, dense bodies and vacuoles; **Figure 12:** High magnification of distal cytoplasm shows tegumental spines at different levels, distal cytoplasm covers spine tips (arrowhead), round bodies,ovoid bodies, opaque bodies, granulated bodies, lucent bodies, mitochondria, infoldings of basal membrane and a vesicle; **Figure 13:** showing apical plasma membrane covers edge of spines, tegumental spines with digitiform tips, panels of distal cytoplasm (arrowheads), rounded bodies, ovoid bodies, road-shape bodies, opaque bodies, lucent bodies, dense trilaminated basal membrane and vacuoles between muscle fibers; **Figure 14:** showing apical plasma membrane coats surface of distal cytoplasm, tegumental spines with digitiform tips homogenous fibrillar medulla and electron-dense base, panels of distal cytoplasm(arrowheads), round bodies, ovoid bodies, cisternae of endoplasmic reticulum, mitochondria, circular muscle fibers, longitudinal muscle fibers, oblique muscle fibers, dense bodies and vesicle; **Figure 15:** High magnification of distal cytoplasm shows round bodies, ovoid bodies, lucent bodies, opaque bodies, microtubules, homogenous fibrillar matrix of the spines, dense trilaminated basal basement membrane, dense bodies, different vacuoles andvesicles.

The fine structure of the tegument of one, three and seven day old C. azimi revealed that corrugations of the outer plasma membrane and the structure of the distal syncytial cytoplasm varied with developmentof the flukes. Inclusion bodies of different size, density and shapes were numerous during early development, but fewer particularly the dense bodies, in adults. The tegumental spines progressively developed and differentiated with the development of the fluke from young juvenile to adult stage. The fibrous basal lamina that was very distinct in one and three day old flukes was less demonstrated in adults, while tegumental muscles became well developed as the fluke develop.

Energy Dispersive X-ray Microanalysis

The level of sulphur (S), calcium (Ca) and phosphorous (P) is determined for spines around the ventral sucker of *C. azimi*in three developmental stages: one, three and seven day old. The spines show high concentrations of sulphur, calcium, and phosphorus in one day old juveniles. The concentration of the three elements decrease gradually with development to reach lowest levels in seven day old flukes except for calcium, that shows increase in level from the day three upon the day seven. In all stages of development, sulphur was the highest in concentration among the three minerals, followed by phosphorus, then calcium.

One way ANOVA revealed high significance differences (0.001) between the three chemical elements in each of the examined stage of development. High significance differences (0.000) were also recorded for each chemical element between the three examined stages of development of the worm (Table 1and Figures 16 and 17).

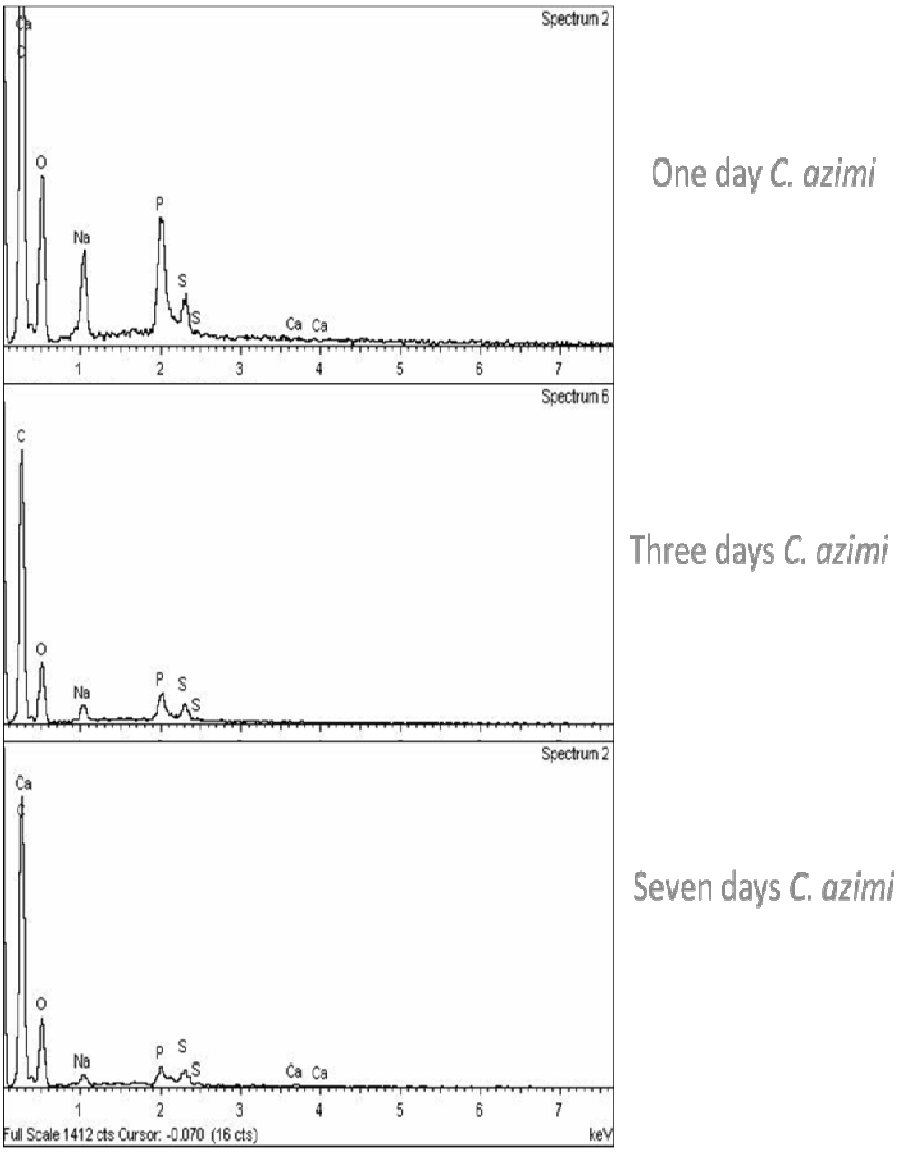


Figure 16: Representative charts showing X-ray microanalysis of the tegumental spines around ventral sucker of 1, 3 and 7 day old, showing chemical elements at their KV peak with the wt. % of each element. The three charts represent example of 9 charts.

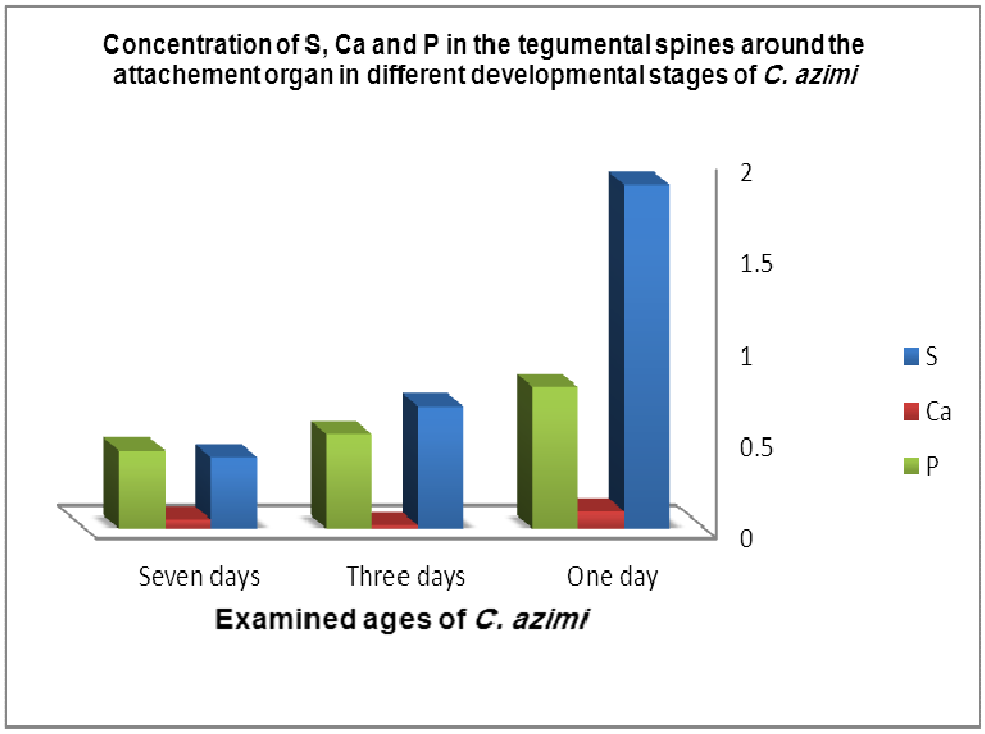


Figure 17: Concentrations of sulphur (S), calcium (Ca) and phosphorus (P) in the tegumental spines around the ventral sucker of *C. azimi* in the three examined stages of development.

Table 1: The mean of weight percent (wt.%) of sulphur (S), calcium (Ca), phosphorus (P) in the tegumental spine around the ventral sucker of C. azimi with mean difference (P value) of EDXA data of the three elements in the three examined stages of development.

Age / Element	One day	Three days	Seven days	P-value
S	1.866 ± 0.595	0.663 ± 0.091	0.386 ± 0.029	0.000**
Ca	0.093 ± 0.0186	0.016 ± 0.001	0.05 ± 0.001	
P	0.77 ± 0.1657	0.513 ± 0.003	0.423 ± 0.008	
P-value	0.001*			

DISCUSSION

The study revealed the fine structural metamorphosis of the tegument on the ventral surface, surrounding the acetabulum of *Cynodiplostomum azimi* during development from early juvenile to adult flukes. The study also showed variations in the sulphur, calcium and phosphorus content of the tegumental spines accompanying development. Fine structural variation accompanying development of *C. azimi* from juvenile to adult was represented by the study of one, three and seven day old *C. azimi*. Corrugations of the apical plasma membrane which increase the surface area needed to enhance substance exchange between the parasite and its environment, showed variations in different areasof the tegument. In juveniles, tegumental corrugations was well revealed; while in adults, the distal cytoplasm appeared as panels interrupted by protruded tegumental spines, the extent of tegumental corrugations varied in different areas.

Variations were also observed in the cytoplasmic inclusions of the distal cytoplasm, as well as the spine density and structure. Inclusion bodies of different size, density and shapes and vesicles were overcrowded during early development, but fewer in the later stages of development, particularly the dense bodies that seems to incorporate in spine formation in the early stages. It was previously reported that inclusion bodies in digenean distal cytoplasm originate from Golgi complex of the perikarya and pass to

the distal cytoplasm via the trabeculae (Bogitch, 1971, Matricon-Gondran, 1971). The suggestion that electron dense bodies participate in the formation of spines, explains their elaborate presence in developing juveniles (Burton, 1964, Matricon-Gondran, 1971). Furthermore, dense bodies were believed to be associated with the emergence and maintenance of the protective outer granular layer of the tegument (Hockley and McLaren, 1973, Lumsden, 1975).

Vesicles particularly those containing inclusions, might be pinocytic or phagocytic in function, since similar structures have been suggested to participate in the absorption and secretion of some materials (Min etal., 1995). Microtubules which were found solitary and in groups are believed to function as a support and transport system (Ghadially, 1988). It is well documented that the digenean tegument plays a vital role in absorption of nutrients, secreation and discharge of waste products (Lumsden, 1975). The fibrous basal lamina which probably plays a supportive function in juveniles was very distinct in one and three day old flukes, but less demonstrated in adults, since it is replaced by the well-developed tegumental muscles in adults. The structural features of the tegument and spines of juvenile and adult *C. azimi* coincide with the description of Khalil (1990 a, b) using SEM and Abo Shafey et al. (1992) using TEM respectively.

Khalil (1990 a) reported that the tegument covering the acetabulum of both juvenile and adult flukes was bare of spines; additionally, in 1990b the author reported that the tegument covering the ventral concavity in the area between the oral sucker and acetabulum was corrugated with irregularly arranged rounded spines in metacercariae; broad indented spines in juveniles; and numerous, flattened and indented spines in adults.

The present study revealed that tegumental spines progressively developed and differentiated with the development of the fluke from young juvenile to adult and its number was increased in adult flukes than in juveniles. Lumsden (1975), Bennet (1975); Font and Wittrock (1980), Fried and Fujino (1984) and Khalil (1990 b) suggested that the morphology and distribution of the tegumental spines vary according to diverse factors including the habitat of parasites, their migratory behavior in the host and the degree of developmental status. Additionally, Khalil (1990 b) suggested that variations in spine topography reflects different functions and referred to Dawes (1963) and Lee et al. (1987) who reported that tegumental spines play a role in abrading host tissue, which in turns cause epithelial hyperplasia and at the same time provides the developing flukes with highly nutritional media. Moreover, Srisawangwonk et al. (1989) suggested that the pectinate spines may support anchorage of the fluke to their intestinal mucosa. Furthermore, Khalil (1990a) and AbouShafey et al (1992) suggested that as the worm develops, the whole ventral concavity with the armed ventral surface, ventro-lateral margins and tribocytic organ form an adhesive suction cup that constitute the main attachment organ for anchorage, while the structure of the aspinose small acetabulum would not support an anchorage function. This suggestion is supported by the present finding of elaborate spines on the ventral surface around the ventral sucker, indicating that as the worm develops spines of the ventral surface becomes equipped for anchorage in certain stages of development.

In the present study the concentration of the three elements (sulphur, calcium and phosphorous) was higher in the spines covering the ventral tegumental surface around the acetabulum of one day old juveniles than that in adult flukes. This unexpected finding may be related to the fact that in young flukes, where the tribocytic organ is poorly developed, the ventral concavity of the body acts as a suction cup which helps the worm to attach to the host's mucosa, therefore the high amount of the studied elements may support the hardness and flexability of the developing spines. Later, in adult stage where the tribocytic organ and the body musculature become well equipped for the adhesive function, the anchorage function of the ventral spines lessens and there become less need for more elements to support the development of more spines around the ventral sucker. This suggestion is supported by previous studies on development, fine structure and chemical nature of the adhesive structures of *C. azimi* (Khalil 1990a, Abou Shafey et al, 1992). Another probable explanation for the high concentration of elements in juveniles is that the presence of elements at high concentration provides anelemental pool of material for the developing spines, while after development the excess elements are excreted.

Sulphur and calcium has been incorporated in anchorage structures of different parasites. High amounts of sulphur in the anchors, hooks and bars and significant amounts of Ca in the anchor of *Gyrodactylus* spp. (Monogenoidea) was related to the collagen like scleroproteins which contain substantial amount of sulphur-bearing amino acids (Kayton, 1983). Likewise, it was reported that calcium and phosphorous in the hooks of the proboscis of Acanthocephala form a rigid calcium and phosphorous complex similar to the enamel of mammalian teeth (Heckmann et al, 2012a). The high sulphur content in the spines of all stages of development confirms the suggestion that sulphur is polymerized into a complex protein with disulfide bonds (Heckmann et al. 2012a). Heckmann et al. (2012a) referred to the important role of disulfide bonds in the stability of protein which is formed between the thiol groups of the amino acids and that cystiene and cystine are probably the main sulphur containing amino acids for acanthocephalan hooks. Radwan et al. (2012) suggested that this may apply for digenean spine.

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